Colorimetric Detection of the Tuberculosis Complex Using Cycling Probe Technology and Hybridization in Microplates

ABSTRACT

Cycling probe technology (CPT) is a simple signal amplification method for the detection of specific target DNA sequences. CPT uses a chimeric DNA-RNA-DNA probe that is cut by RNase H when bound to its complementary target sequence. In this study, a hybridization assay was developed to detect biotinylated CPT products that result from the amplification of a Mycobacterium tuberculosis complex sequence. The chimeric probe was specifically designed to avoid the formation of secondary structures. The chosen capture probe was perfectly complementary to and was the same size as OL2, one of the two CPT products. The assay was based on the observation that a long sequence, such as the initial probe, was destabilized when bound to a small capture probe as a result of steric hindrance. The capture probe preferentially bound OL2 rather than the long initial probe. We added a prehybridization step with a helper DNA to enhance this discrimination between the two sequences. Colorimetric detection was performed using a peroxidase-streptavidin conjugate. After optimization, the non-isotopic hybridization assay allowed the detection of around 10 amol of target DNA. Besides being faster and easier to perform, this detection method was compared to electrophoresis separation and gave similar results.

INTRODUCTION

Cycling probe technology (CPT) is an isothermal signal amplification method for the detection of specific target DNA sequences (Figure 1). A chimeric probe, DNA-RNA-DNA and thermostable RNase H are the two main components of this assay. In the presence of the target sequence, a DNA/RNA hybrid is formed and RNase H specifically catalyzes the cleavage of the RNA portion of the hybrid. Since cleaved fragments are small, they dissociate spontaneously from the target sequence at the reaction temperature. The target is then recycled and available for hybridization with another probe; the reaction is inherently cyclic without external manipulations (8,19). The labeled, cleaved probes accumulate with time and are detected as described here. Since there is no target amplification, carryover contaminations are minimized (1).

Usually, the chimeric probe is labeled with radioisotope $[^{32}\text{P}]$, and the cleaved probe percentage is evaluated by electrophoresis on a polyacrylamide gel (1,7,9,12,13). This method is time consuming, limits the number of samples that can be simultaneously analyzed and is not easily automated. Furthermore, the use of radioisotopes such as $[^{32}\text{P}]$ is not optimal because of safety reasons (6) and for their short half-life that requires frequent probe preparation (16). Nonradioisotopic detection has already been developed for the detection of the meca gene (2), but it was a subtractive assay that was limited to a dynamic range of less than one order of magnitude.

The purpose of this study was to develop a nonradioactive detection method by hybridizing the cleaved probes on capture probes covalently bound on a microplate (21,22). The challenge was to specifically detect the cleaved probe while the initial uncut probe was still present in solution. The steric hindrance of the support should favor the hybridization of the cut probe. Moreover, a prehybridization step was added in the presence of a helper probe to further prevent the hybridization of the uncut probe on the capture probe (Figure 1). The biotinylated probes were then detected by colorimetry through a streptavidin-peroxodase complex.

The method was developed for the detection of the tuberculosis complex. In fact, the presence of bacteria from this complex (Mycobacterium tuberculosis, M. bovis, M. bovis BCG, M. microti and M. africanum) requires a rapid and sensitive diagnosis that cannot be provided by classical microbiological methods that rely on morphological, biochemical and growth properties (8,17,18). A fast diagnosis is one of the requirements for the effective control of tuberculosis (20).

MATERIALS AND METHODS

Radioisotopic Labeling of Probes

The probes were labeled with ATP $[^{32}\text{P}](\text{Dupont Denemours, Brussels, Belgium})$ by incubation with T4 polynucleotide kinase (Roche Molecular Biochemicals, Mannheim, Germany) for 30 min at $37^\circ\text{C}$. The addition of 0.25 M EDTA stopped the reaction. Labeled probes were purified on a G25 Sephadex® column (Roche Molecular Biochemicals, Mannheim, Germany).

Covalent DNA Immobilization in Microwells

The capture DNA probe was covalently attached to the microwells by the formation of a phosphoramidite bond between the $5^′$ terminal phosphate group and the aminogroup of the poly styrene microwells (15,21,22).

The capture probe (25 pmol) ($5′$-TCGGCACGGACACGGC TCTGAT-3′) (Eurogentec, Seraing, Belgium) was heated for 10 min at $94^\circ\text{C}$ and mixed with 10 mM 1-methylimidazole (pH 7.5). The solution was then dispensed into the microwells (75 µL/well) (Covalink-NH; Nalge Nunc, Roskilde, Denmark). Forty micromoles of EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide] (Sigma, St. Louis, MO, USA) were dissolved in 10 mM 1-methylimidazole and 25 µL were added to each well. The plates were incubated at $50^\circ\text{C}$ for 5 h and subsequently washed three times for 5 min with 0.4 M NaOH/0.25% Tween® 20. After a last wash with water, the plates were dried and kept at $4^\circ\text{C}$.

CPT Assay

The chimeric probe was chosen within the mycobacterial fragment Mt308; this sequence is specifically present in mycobacteria belonging to the tuberculosis complex and found as a single copy in the genome (18,19).

Two secondary-structure prediction
programs were used to design the chimeric probes. The first was Oligo 6, which predicts the stability of probes and displays hairpins of more than 3 bp (5). The second, DNA fold, was developed by M. Zucker (10) for RNA and adapted to DNA by Nielsen (14). It is based on free-energy minimization and predicts the most stable secondary structures present at different temperatures. Naview, the program discussed by Bruccoleri and Heinrich (23), allowed drawn forms of the predicted structures.

The chimeric probes, OLM1 and OLM1b, were synthesized by ID Biomedical (Bothell, WA, USA). OLM1 was 39 bases in length with three ribonucleotides contained within the sequence 5′-d(ATGTCAGAGCCGTGTCCGTGCCG)r(AAG)d(TGTTACC CGAGT)-3′, and OLM1b was biotinylated at the 5′ terminus. The complementary synthetic target sequence was 5′-ACTCGGGGTAACAC- TTCGGCACCGAAGCTGACAG-3′ (Eurogentec).

The reaction mixture (10 µL final) contained cycling buffer [50 mM Tris-HCl (pH 8.1), 8 mM MgCl$_2$ and 0.025% Triton X-100], 100 fmol chimeric probe, 5.7 pmol RNase H (ID Biomedical) and different amounts of target sequence. The mixture was incubated for 30 min at 70°C.

In each experiment, two reaction controls, C1 and C2, were carried out without target sequence, where C1 did not contain RNase H and C2 did. C2 was essential to evaluate the nonspecific degradation of the chimeric probe. The CPT reaction was stopped by adding 5 µL loading buffer (8 M urea, 0.1 M EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol).

Amplification Product Analysis on a Polyacrylamide Gel

The CPT products were separated by electrophoresis on a 20% polyacrylamide gel in denaturing conditions (7 M urea) at 200 V for 3 h. The amount of the respective products was estimated by integration of the different bands (intact and digested probe) with an Instant Imager (Packard, Downers Grove, IL, USA).

Amplification Product Analysis by Hybridization

For the prehybridization step, the CPT reaction was stopped by heat denaturation at 94°C for 10 min. Then, 10
pmol in 10 µL helper DNA (5′-ACTCCGGGTAAACACTTCGGCACCGA-3′) (Eurogentec) and 50 µL hybridization buffer (0.5× SSC and 100 µg/L ssDNA), both purchased from Sigma, were added and incubated for 1 h at 45°C in microtubes.

For the hybridization step, the wells bearing the DNA capture probe were incubated with 0.2 M NaOH for 5 min and then washed with 0.2× SSC. The prehybridization mixture was transferred to the wells and incubated for 2 h at 50°C. The wells were then washed three times for 5 min with 0.4× SSC warmed to 45°C. For detection when using the radioisotopic probe, the wells were removed and radioactivity was counted in a scintillation counter (Beckman Coulter, Fullerton, CA, USA). When colorimetric detection was used, wells were washed once with blocking buffer to decrease the background by saturating the surface with proteins [0.1% Gloria milk powder in buffer 1:0.1 M maleic acid, 0.15 M NaCl (pH 7.5)], and 100 µL streptavidin-peroxidase conjugate (Medgenix, Fleurus, Belgium) diluted 1000× in blocking buffer was dispensed into wells. After a 45-min incubation at room temperature, the wells were washed three times for 5 min with buffer 2 (buffer 1 plus 0.3% Tween 20) and once for 5 min with buffer 1. The substrate tetramethyl benzidine (TMB) (Medgenix) was added (100 µL/well) and incubated for 15 min in the dark. The addition of 100 µL acidic solution (Medgenix) stopped the colorimetric reaction. The absorbance was then measured at 405 nm. The values were corrected for the background of the plastic by measuring this absorption at 655 nm.

RESULTS AND DISCUSSION

Chimeric Probe Design

The chimeric probe OLM1 was selected within the Mt308 sequence and consisted of 33 matching bases flanked by six nonspecific bases at the 5′ end. This six-base-stretch was part of the fragment hybridized on the capture probe. The presence of secondary structure in OLM1 was checked using Oligo 6 and DNA fold prediction programs.

In our first attempt, we observed high C2 values from the spontaneous cleavage of the probe in the presence of RNase H (data not shown). This cleavage was the result of secondary structures within the probe. To eliminate this nonspecific cleavage, the number of RNA bases within the chimeric probe was reduced from four to three bases. The use of three RNA bases is known to slightly reduce the efficiency of the CPT (11); in our case, it was necessary to decrease the cleavage of the initial probe. Following the action of RNase H, OLM1 was cleaved into two small probes: OL2 on the 5′ side and OL3 on the 3′ side of the initial probe. After amplification, the OL2 fragment was the only one labeled.

Detection by Hybridization on a Microplate

A unique feature of this work was investigating the possibility of discriminating between OL2 and OLM1 by hybridization on a capture probe linked at the bottom of the well. Theoretically, the two probes could hybridize because they are both complementary to the capture probe. However, OLM1 is longer than OL2, and when fixed on the capture probe, a steric hindrance will create an instability. We decided to use this physical hindrance to discriminate between the OL2 and OLM1 sequences for this hybridization step.

Capture Probe Immobilization on Microwells

The capture probe immobilization was a crucial step in influencing the sig-

Figure 2. Titration of the amount of OLM1 probe in the CPT hybridization assay. The amount of DNA target (T) was 1 fmol. Prehybridization was performed with 10 pmol helper DNA. Two controls were added: C1 included the probes but no target and no RNase H; C2 included the probe and RNase H but no target. Results were expressed as the amount of CPT products hybridized to the capture probe.

Figure 3. Histogram showing the detection limit obtained by CPT amplification and colorimetric detection. CPT was performed with 100 fmol OLM1b. Targets were serially diluted as follows: 1 fmol (T1), 100 amol (T2), 10 amol (T3) and 1 amol (T4). The prehybridization step was performed with 10 pmol helper DNA. Two controls were added: C1 included the probes but no target and no RNase H; C2 included the probe and RNase H but no target. Results were expressed as the OD at 405–655 nm.
nal-to-noise ratio and affecting the sensitivity of the assay (4,6). Covalent binding of DNA on microwells was obtained by fixation on the 5′-end-activated amino group on the plastic (15,22). Variable amounts (10, 25 and 50 pmol) of 5′-end-radiolabeled capture probes were fixed to define the amount that gives the best fixation yield. This immobilization reaction was performed for 5 h at 50°C in 10 mM methylimidazole buffer (pH 7.5) with 40 mM EDC. Nonspecific fixation was estimated by carrying out the reaction in the absence of EDC.

The amount of bound capture probe increased with the starting amount up to a maximum level when 25 pmol was added (data not shown). Under these conditions, 0.5 pmol capture probe was fixed on the solid support, and the nonspecific binding was quite low. Therefore, 25 pmol capture probe was selected to carry out the fixation process on the microplates.

**OL2 Detection by Hybridization on the Microplate**

During the CPT assay, the OLM1 probe is cleaved into two small fragments, OL2 and OL3. OL2 is labeled and detected after hybridization on the capture probe fixed in the wells. Hybridization conditions were first optimized using purified radiolabeled OL2. Different times and temperatures were tested. Since the principle of this differential assay is based on the destabilization of the OLM1 hybridization by steric hindrance, the study of the temperature and the stringency of the washing conditions was a crucial step. The most effective washing condition was three times with 0.4× SSC at 45°C for 5 min (data not shown). Under these conditions, we obtained a good yield for the hybridization of the OL2 sequence with a minimal amount for OLM1. However, the discrimination was not absolute.

We decided to perform a prehybridization step in the presence of a small blocking sequence (helper DNA). The optimal conditions used to block OLM1 binding were 10 pmol helper DNA incubated for 1 h at 45°C. The helper probe sequence hybridized to a part of the OLM1 RNA sequence, but RNase H was inactivated at that time, so there was no cleavage.

Finally, the CPT conditions were adapted to the detection of OL2 by hybridization. We found that increasing the OLM1 amount to 100 fmol led to an increase in OL2 detection (Figure 2). Once hybridization conditions were optimized with OLM1, we performed the CPT with the biotinylated probe OLM1b and compared the sensitivity of this hybridization assay with classical radioisotopic detection by electrophoresis on a polyacrylamide gel.

**Detection Limit of the CPT Assay**

A colorimetric hybridization assay was performed on the CPT products (Figure 3), and results were compared to those obtained after radioisotopic hybridization assay or electrophoretic analysis of the CPT products (Table 1). Different parameters such as the detection limit and the C1:C2 ratio were compared.

In the colorimetric detection after hybridization, a high signal was obtained for a target concentration of 1 fmol and 100 amol (Figure 3). The signal then decreased and reached, at 1 amol, a value close to the C2 control. The C1 value represented the detection of the OLM1b substrate probe alone, while the C2 value also took into account the nonspecific cleavage of the probe by RNase H. We could conclude that, for this assay, the detection limit was around 10 amol of target DNA (10^7 target copies), which corresponded to the limit obtained for mecA gene detection (7). The dynamic range of this assay reached nearly two logs, which is higher than the nonradioisotopic system described elsewhere (7).

The lower sensitivity in hybridization was partially a result of higher signals obtained in the controls. On a polyacrylamide gel, the cleaved probe migrated faster and could be quantified without interference from the starting probe. However, when detected by hybridization, there is little binding of OLM1, even in the optimized conditions. This effect can be observed by a higher C1 value and a decrease in the C1:C2 ratio (Table 1). When added at the same concentration, the ratio between the hybridization of OLM1b and OL2 was 11.7. This means that out of the 100 hybridized probes, nine were
OLM1b. This OLM1b binding increased both C1 and C2 values because a 50-fold higher amount of OLM1b was used in this assay compared with the electrophoretic separation assay.

Compared to electrophoretic separation, the differential hybridization proposed here is slightly less sensitive, quite simple to perform and can be part of an automation process with a robot adapted for 96 wells. The future development of the colorimetric detection of CPT products in microwells by the method described above involves its application in diagnostics. However, some problems could occur if one wants to apply CPT to clinical samples because they may contain nonspecific RNases. Moreover the high amounts of human genomic DNA affect the yield of the probe’s cleavage. It has been shown by Modrusan et al. (12) that the presence of genomic DNA in CPT resulted in high background and was inhibitory. Addition of spermidine and EGTA into the CPT reaction greatly inhibitory. Addition of spermidine and EGTA into the CPT reaction greatly improved the assay (12).

The CPT assay is rapid and does not require an expensive instrument. Once its adaptation to clinical samples is made possible, it may provide a real alternative to the PCR-based assays in molecular microbiology.

REFERENCES


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S. Warnon, N. Zammatteo, I. Alexandre, C. Hans and J. Remacle
Facultés Universitaires Notre-Dame de la Paix Namur, Belgium

Application of DNA Topoisomerase-Activated Adapters to Riboprobe Synthesis

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ABSTRACT

Topoisomerase-activated adapters for rapid incorporation of the T7 promoter into PCR products were made by hybridizing synthetic oligonucleotides and activating vaccinia DNA topoisomerase I. The adapters were used to incorporate the T7 promoter sequence into PCR products amplified from cDNA and genomic DNA. Modified PCR products were used as templates to synthesize digoxigenin-labeled sense and cRNA probes by in vitro transcription with phage T7 RNA polymerase. The red/green cones were labeled by the antisense probe, but no specific signal was produced by the sense probe. These results demonstrate that topoisomerase-activated adapters provide a powerful and convenient tool for the rapid modification of PCR products.

INTRODUCTION

Recent advances in gene discovery technology and the human genome project have revealed thousands of new sequences, which are being characterized for tissue- or disease-specific expression. These analyses involve Northern blotting, RNase protection and in situ hybridization techniques that often rely on the use of riboprobes generated from DNA templates by in vitro transcription (IVT). Traditional approaches to template preparation for IVT include subcloning the DNA of interest into a plasmid vector with an appropriate RNA polymerase promoter (6) or incorporating the promoter sequences during PCR amplification with primers carrying the promoter sequence at their 5′ ends (5).

While both techniques seem to be reliable and have certain advantages, the first one requires lengthy and laborious steps to transform bacterial cells and identify the right recombinant clones, plasmid DNA purification and linearization. The second one requires a synthesis of primers with 5′-end extensions containing RNA polymerase promoters for every DNA of interest, in addition to regular gene-specific primers, a method that may be time and cost inefficient over the long run.

This report introduces a novel technique to quickly generate templates for IVT from PCR products. The method is based on the use of topoisomerase-activated adapters containing the promoter for T7 RNA polymerase. The adapters are hybridized oligonucleotides that have been cleaved and activated by vaccinia DNA topoisomerase I. This enzyme has unique properties that allow cleavage of dsDNA at a CCCTT recognition site, form a covalent bond with the substrate and religate it to an acceptor DNA through a 5′-hydroxyl group.